INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: ASSAY FOR MULTIPLE ANALYTES WITH CO-IMMOBILIZED LIGANDS
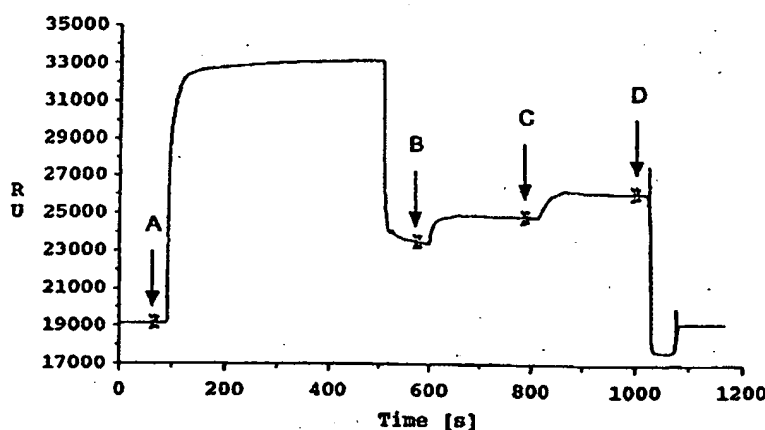
(57) Abstract

A method of assaying for at least two different analytes in a fluid sample, wherein each analyte is determined by detecting or measuring a mass change at a solid sensing surface caused directly or indirectly by the analyte, comprises the steps of co-immobilizing to the same sensing surface different catching molecules each capable of specifically binding to either a respective analyte or a respective analyte analogue or analyte specific binding partner added to the sample, and either (i) after contacting the surface with the sample, determining the binding of each different analyte, analyte analogue or specific binding partner to the respective catching molecule by sequentially contacting the obtained surface with respective specific reagents to said analytes, analyte analogues or specific binding partners, or (ii) sequentially contacting the immobilized surface with sample portions containing either different specific binding partners to the respective sample analytes or different analyte analogues to determine the binding of each specific binding partner or analyte analogue to the respective immobilized analyte or analyte analogue or specific binding partner, respectively.

Assay for multiple analytes with CO-immobi-
lized ligands.

5   The present invention relates to an improvement in
assay methods of the type where the presence of an analyte
is detected by contacting the sample with a solid sensing
surface, and determining a mass change at the surface
caused directly or indirectly by the analyte in the sample.
        One class of methods for determining such changes in
the mass at a sensing surface utilizes evanescent wave
10   sensing at an optical surface. Evanescent wave sensing
technology based upon surface plasmon resonance,
hereinafter SPR, has recently been developed for inter alia
immunoassay methods. The phenomenon of SPR is well known.
In brief, SPR is observed as a dip in intensity of light
15   reflected at a specific angle from the interface between an
optically transparent material, e.g. glass, and a thin
metal film, usually silver or gold, and depends on among
other factors the refractive index of the medium (e.g. a
sample solution) close to the metal surface. A change of
20   refractive index at the metal surface, such as by the
adsorption or binding of material thereto, will cause a
corresponding shift in the angle at which SPR occurs. To
couple the light to the interface such that SPR arises, two
alternative arrangements are used, either a metallized
25   diffraction grating (Wood's effect), or a metallized glass
prism or a prism in optical contact with a metallized glass
substrate (Kretschmann effect). For further details on SPR,
reference is made to our WO 90/05295. In an SPR-based
immunoassay, a ligand may be bound to the metal surface,
30   and the interaction thereof with an analyte of an aqueous
sample in contact with the surface is monitored.
        In our above-mentioned WO 90/05295 and our WO 90/05305
there are described an SPR-based biosensor system and a
sensor unit, respectively, permitting simultaneous or
35   sequential measurement of several different analytes by
providing several sensing or detection surface areas
defined on the metal surface, each sensing area supporting
a different ligand and enclosed in a respective flow cell.

The separate flow cells are arranged to be passed by the
sample flow either in parallel or in series.

Similarly, US-A-4,889,427 discloses a method and
apparatus for the simultaneous or sequential measurement of
5 several different analytes by providing several separate
metal strips arranged in a single flow cell to be passed by
the sample flow, each metal strip supporting a different
ligand, such as an antibody.

The optical and mechanical design of the systems
10 required for performing such measurements is relatively
complex. The present invention therefore seeks to reduce
the complexity of such systems for simultaneous
determination of two or more analytes.

In accordance with the present invention this is
15 accomplished by co-immobilizing the different specific
ligands or reagents in a single surface area and, after
contacting the sample with the surface area, directly or
indirectly detecting the different analytes sequentially.
Co-immobilization in the present context means that these
20 different specific ligands or reagents are randomly or
orderly immobilized to the single surface area.

The present inventive concept makes use of the fact
that the specific response for surface concentration, or
mass, detecting devices, such as surface plasmon resonance
25 detection, is in principle independent of the size of the
detecting area, at least down to the diffraction
limitations of the optical system. This is in contrast to
absolute measuring devices such as used in ordinary solid
phase assays where the specific response is area dependent.
30 This independence of area size for the surface
concentration detecting device will thus readily allow
reduction in the number of detection areas by co-
immobilization as proposed above.

As is readily understood, such co-immobilization
35 permitting the determination of two or more different
analytes in a single sensing area will greatly reduce the
complexity of the optical and mechanical, including the
microfluidic design of the analytical system.

The present invention therefore provides a method of qualitatively or quantitatively assaying for at least two different analytes in a fluid sample, wherein each analyte is determined by detecting or measuring a mass change at a
5   solid sensing surface caused directly or indirectly by the analyte, which method is characterized by co-immobilizing to the same sensing surface different catching molecules each capable of specifically binding to (depending on the assay principle) either a respective analyte or a
10 respective analyte analogue or analyte specific binding partner added to the sample, and either (i) after contacting the surface with the sample, determining the binding of each different analyte, analyte analogue or specific binding partner to the respective catching
15 molecule by sequentially contacting the obtained surface with respective specific reagents to said analytes, analyte analogues or specific binding partners, or (ii) sequentially contacting the immobilized surface with sample portions containing either different specific binding
20 partners to the respective sample analytes or different analyte analogues to determine the binding of each specific binding partner or analyte analogue to the respective immobilized analyte or analyte analogue or specific binding partner, respectively.
25   As used herein the term analyte analogue means either a molecule having a similar binding reactivity against an immobilized ligand or capturing molecule as the analyte, or the analyte conjugated with another molecule which does not change the binding characteristics of the analyte.
30   The term specific binding partner as used herein means a molecule which reacts specifically with a particular molecule, such as an analyte or immobilized capturing molecule.
         The term specific reagent is used herein to denote a
35 reagent used to generate the specific analyte detection signal which correlates to the analyte concentration in the sample.

The method of the invention readily permits
simultaneous measurement of two different analytes, but
three or even more analytes may be determined without
affecting the precision of the concentration determination.

5   The immobilized capturing molecules may be low
molecular weight (LMW) as well as high molecular weight
(HMW) molecules or a mix of LMW and HMW molecules.

The co-immobilization concept is, as appears from the
above definition of the method of the invention, compatible

10   with several different per se conventional assay formats or
principles for concentration determination, such as e.g.
sandwich assay, inhibition assay, displacement assay,
competitive assay or combinations thereof.

For instance, a sandwich assay may be performed by

15   immobilizing different capturing molecules, such as
antibodies, to the sensing surface which are capable of
reacting with the respective analytes. Each analyte must in
this case be bifunctional, i.e. exhibit a second binding
site in addition to that binding to the corresponding

20   immobilized capturing molecule. Reagents which are each
capable of binding specifically to a respective analyte,
such as antibodies, are then added in sequence, and the
binding of one reagent to its respective analyte is then
determined before the next reagent is added. The amount of

25   reagent bound is proportional to the concentration of the
corresponding analyte in the sample.

A competitive assay may be performed by adding analyte
analogues to the sample and have the analytes compete with
the analyte analogues for the binding to respective

30   capturing molecules co-immobilized to the surface. The
amount of each analyte analogue bound to the surface is
then determined by sequentially contacting the surface with
reagents, such as antibodies, specifically binding to the
respective analyte analogues. The determined amount of

35   analyte analogue is inversely proportional to the
concentration of the corresponding analyte in the sample.
The analyte analogue may, for example, be a HMW molecule
such as an antibody, but may also be a LMW molecule

conjugated to a LMW or HMW molecule, such as e.g. bovine
serum albumin, to which the specific reagent is directed.

In the case of an inhibition assay, analytes or
analyte analogues may be co-immobilized on the sensing
5   surface. Specific binding partners to the analytes are then
added to the sample, and after contacting the sample with
the sensing surface, the bound specific binding partners
are determined by sequentially contacting the surface with
specific reagents capable of specifically reacting with the
10   binding partners. The latter may, for example, be
antibodies conjugated with respective different molecules
against which the specific reagents may be directed. As is
readily understood, the determined amount of each specific
binding partner is inversely proportional to the
15   concentration of the respective analyte in the sample.

Alternatively, specific binding partners for the
respective analytes may be added to separate portions of
the sample containing the analytes. By sequentially
contacting the different sample portions with the surface
20   having analytes or analyte analogues co-immobilized
thereto, the binding of each specific binding partner may
be determined as inversely indicating the concentration of
the corresponding analyte in the sample.

A displacement assay may be performed by co-
25   immobilizing analytes or analyte analogues on the sensing
surface and saturating substantially all immobilized
analytes or analyte analogues by binding specific binding
partners thereto. The surface is then contacted with the
sample, the analytes in the sample causing the specific
30   binding partners to be partially removed by either
competing for the binding to the analytes or binding
otherwise to the specific binding partners such that they
are released from the surface. The amount of each remaining
specific binding partner, which is inversely proportional
35   to the respective analyte concentration in the sample, is
then determined by sequential addition of specific reagents
directed against the respective specific binding partners.
Each specific reagent used must, of course, be directed

against another binding site on the corresponding specific binding partner than that participating in the binding to the respective surface immobilized analyte or analyte analogue.

5   In all the above described assay formats, the detection responses may, if desired, be increased by further sequential additions of additional specific reagents which bind to the respective species bound in the preceding detection step.

10   The contact between the fluid sample medium and the optical surface may be static, or preferably, dynamic, i.e. the provision of the sensing surface in some kind of flow cell.

Suitable sensing surfaces to be used in the present
15   invention are described in our WO 90/05303 which discloses sensing surfaces capable of selective biomolecular interactions and designed to be used in biosensor systems, particularly systems based upon surface plasmon resonance (SPR). These sensing surfaces comprise a film of a free
20   electron metal, preferably silver or gold, having one of its faces coated with a densely packed monolayer of specific organic molecules. To this monolayer a biocompatible porous matrix, e.g. a hydrogel, is bound, which matrix is employed for immobilizing suitable ligands
25   for target biomolecules to be determined by the particular biosensor.

The mass sensing methods for which the present invention may be used are, as mentioned previously, not restricted to SPR methods, but extend to any assay method
30   measuring a mass change at a sensing surface as being indicative of the presence of an analyte. Such methods generally include reflection optical methods, both internal and external, for example, ellipsometry and evanescent wave spectroscopy, the latter including Brewster angle
35   reflectometry, critical-angle reflectometry, evanescent wave ellipsometry, scattered total internal reflection (STIR), optical waveguide sensors, etc.

Situations where it is of interest to analyze more
than one analyte and to which the present invention thus
may be applied are, for example, clinical situations
requiring the analysis of more than one analyte to make a
5   correct diagnosis or decision, and food as well as
environmental analyses where it is of importance to analyse
more than one analyte at a time to get a general picture of
the situation. Exemplary of such clinical situations are
myocardial infarction, fertility examination and
10   transplantation surgery. The determination of antibiotics
in milk is an example of food analysis, and the
determination of pesticides in water may be mentioned as an
example of environmental analysis.

In the following, the invention will be described in
15   more detail by non-limiting examples, reference being made
to the accompanying drawings where:

Fig. 1 is an SPR-sensor diagram showing the co-
immobilization of a monoclonal antibody specific for CK-MB
and a monoclonal antibody specific for myoglobin to a
20   sensing surface;

Fig. 2 is a corresponding diagram to Fig. 1 showing
the analysis of a sample containing creatine kinase MB (CK-
MB) and myoglobin using the sensing surface with co-
immobilized monoclonals against CK-MB and myoglobin,
25   respectively, in Fig. 1;

Fig. 3 is a corresponding diagram to Fig. 2 showing
the analysis of a sample containing ß2-microglobulin and
IgE using a sensing surface with co-immobilized monoclonals
against ß2-microglobulin and IgE, respectively;

30   Fig. 4 is a diagram showing standard curves for ß2-
microglobulin obtained when analyzing ß2-microglobulin on a
single-immobilized anti-ß2-microglobulin surface and on a
co-immobilized anti-ß2-microglobulin/anti-IgE surface,
respectively;

35   Fig. 5 is a diagram showing the precision profiles for
ß2-microglobulin in the analysis in Fig. 4;

Fig. 6 is a diagram showing standard curves for IgE
obtained when analyzing IgE on a single-immobilized anti-

IgE surface and on a co-immobilized anti-IgE/anti-β2-
microglobulin surface, respectively; and

Fig. 7 is a diagram showing the precision profiles for
IgE in the analysis in Fig. 6.

In the following Examples, the measurements are
performed on a commercial SPR-based biosensor instrument
(BIAcore™) and commercial sensing surfaces (Sensor Chip™
CM5) (both marketed by Pharmacia Biosensor AB, Uppsala,
Sweden).

EXAMPLE 1

A. Co-immobilization of monoclonal antibodies on sensing
surface

Immobilization on a sensing surface of a monoclonal
antibody specific for CK-MB and a monoclonal antibody
specific for myoglobin was performed in the biosensor
instrument in the following manner:

A continuous flow of HBS (10 mM Hepes buffer, 0.15 M
NaCl, 3.4 mM EDTA, 0.05 % Tween), pH 7.4, over the sensing
surface was maintained at 5 µl/min. A fraction of the
carboxyl groups on the sensing surface was activated to
form reactive N-hydroxysuccinimide esters by injecting into
the instrument 35 µl of a solution containing 0.2 M 1-
ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride
(EDC) and 0.05 M N-hydroxysuccinimide (NHS) in water. 35 µl
of the antibody solution containing 50 µg/ml of a
monoclonal antibody specific for CK-MB (obtained from
BiosPacific, Emeryville, California, U.S.A.) and 50 µg/ml
of a monoclonal antibody specific for myoglobin (obtained
from the Institute of General and Molecular Pathology,
Tartu State University, Tartu, Estonia) in 10 mM sodium
acetate, pH 5.0, were then injected. A buffer with a pH
below the pI of the antibody will give a positive net
charge of the protein, and at low ionic strength the
antibodies will preconcentrate to the remaining negatively
charged carboxyl groups on the surface via electrostatic
attraction giving a high antibody concentration in the
matrix. The preconcentration allows fast immobilization
with low amount of antibodies. Remaining reactive ester

groups were deactivated by injection of 35 μl of 1 M
ethanolamine hydrochloride, pH 8.5. The sensorgram obtained
is shown in Fig. 1 (response in resonance units, RU,
plotted versus time in seconds). The response signal was
5   evaluated at two levels: 20 seconds before the injection of
EDC/NHS (A) and 9 minutes after the injection of
ethanolamine (B). B minus A thus defines the immobilized
amount of the two antibodies.

B. Analysis of plasma samples

10   The analysis of CK-MB and myoglobin at elevated levels
in a plasma sample, using the sensing surface with co-
immobilized antibodies prepared in section A above, was
performed in the following manner:

A continuous flow of HBS (10 mM Hepes buffer, 0.15 M
15   NaCl, 3.4 mM EDTA, 0.05 % Tween), pH 7.4, over the sensing
surface was maintained at 5 μl/min. 35 μl of a plasma
sample containing CK-MB and myoglobin were injected into
the instrument. 4 μl each of second antibodies specific for
CK-MB and myoglobin, respectively, at a concentration of
20   100 μg/ml were then injected in sequence followed by 4 μl
of 10 mM glycine-HCl, pH 2.5. The sensorgram obtained is
shown in Fig. 2 (response in resonance units, RU, plotted
versus time in seconds). The response signal was evaluated
at four levels: 20 seconds before the injection of the
25   sample (A), 20 seconds before the injection of the second
antibody specific for CK-MB (B), 20 seconds before the
injection of the second antibody specific for myoglobin
(C), and 20 seconds before the injection of glycine-HCl
(D). Thus, A defines the baseline, B minus A defines the
30   plasma response, C minus B defines the specific response
for CK-MB, and D minus C defines the specific response for
myoglobin. The analysis time was 18 minutes.

                           EXAMPLE 2

        In the same way as described in Section A of Example 1
35   above, antibodies specific for β2-microglobulin and IgE,
respectively, were co-immobilized to the sensing surface by
injecting 15 μg/ml anti-β2-microglobulin monoclonal (Kabi
Pharmacia AB, Sweden)) and 30 μg/ml anti-IgE monoclonal

(Kabi Pharmacia AB, Sweden). Analysis of β2-microglobulin
and IgE was then performed by maintaining a continuous flow
of HBS (see Example 1) at 5 μl/ml. 35 μl of a buffer sample
containing β2-microglobulin and IgE was injected. 4 μl each

5   of respective second antibodies to β2-microglobulin and IgE
were then injected in sequence followed by 4 μl of 10 mM
glycine-HCl, pH 2.5. The second antibody for β2-
microglobulin was an Ig-fraction of a polyclonal antibody
at 2.5 mg/ml in 10 mM Hepes, pH 7.4. The second antibody

10   for IgE was a mix of two monoclonal antibodies at a
concentration of 50 μg/ml of each in 10 mM Na-acetate, pH
5. The sensorgram obtained is shown in Fig. 3 (response in
resonance units, RU, plotted versus time in seconds). The
response signal was evaluated at four levels: 20 seconds

15   before the injection of the sample (A), 20 seconds before
the injection of the second antibody specific for β2-
microglobulin (B), 20 seconds before the injection of the
second antibody specific for IgE (C), and 20 seconds before
the injection of glycine-HCl. A defines the baseline, B

20   minus A defines the plasma response, C minus B defines the
specific response for β2-microglobulin, and D minus C
defines the specific response for IgE. The analysis time
was 21 minutes.

                          EXAMPLE 3

25   By proceeding correspondingly as in Example 2,
standard curves for β2-microglobulin analysis were obtained
by analyzing β2-microglobulin standards on (i) a co-
immobilized anti-β2-microglobulin/anti-IgE surface and (ii)
a single-immobilized anti-β2-microglobulin surface. The

30   standards containing β2-microglobulin or β2-microglobulin
and IgE were injected in a concentration range from 0.35 to
5.55 nM over the immobilized antibody surface followed by
injection of a polyclonal anti-β2-microglobulin. The second
antibody responses were used to construct the standard

35   curves. The standard curves obtained are shown in Fig. 4,
and the corresponding coefficient of variation in
concentration (CV) or precision profiles [(standard
deviation/mean concentration) x 100] are shown in Fig. 5.

In a corresponding manner, standard curves for IgE
were obtained by injecting standards containing IgE or IgE
and β2-microglobulin in a concentration range from 0.35 to
5.55 nM over the immobilized antibody surface followed by
5   injection of a mix of two monoclonal IgE antibodies. The
second antibody responses were used to construct the
standard curves. The standard curves obtained are shown in
Fig. 6, and the corresponding precision profiles are shown
in Fig. 7.
10   As appears from Figs. 4 to 7, the co-immobilization
did not affect neither the response dynamics nor the
precision in the concentration determination.
         The invention is, of course, not restricted to the
above specifically described embodiments, but many
15   modifications and changes may be made without departing
from the scope of the general inventive concept as defined
in the subsequent claims.

CLAIMS

1.   A method of assaying for at least two different
analytes in a fluid sample, wherein each analyte is
5   determined by detecting or measuring a mass change at a
solid sensing surface caused directly or indirectly by the
analyte, **characterized** by co-immobilizing to the same
sensing surface different catching molecules each capable
of specifically binding to either a respective analyte or a
10 respective analyte analogue or analyte specific binding
partner added to the sample, and either (i) after
contacting the surface with the sample, determining the
binding of each different analyte, analyte analogue or
specific binding partner to the respective catching
15 molecule by sequentially contacting the obtained surface
with respective specific reagents to said analytes, analyte
analogues or specific binding partners, or (ii)
sequentially contacting the immobilized surface with sample
portions containing either different specific binding
20 partners to the respective sample analytes or different,
analyte analogues to determine the binding of each specific
binding partner or analyte analogue to the respective
immobilized analyte or analyte analogue or specific binding
partner, respectively.
25

2.   The method according to claim 1, **characterized** in that
it comprises co-immobilizing to the sensing surface
different capturing molecules each capable of binding to a
respective analyte, contacting the sample with the
30 immobilized surface, sequentially reacting the bound
analytes with different reagents specifically reacting with
the respective analytes, and determining the binding of
each reagent to the respective analyte.

35 3.   The method according to claim 1, **characterized** in that
it comprises adding analyte analogues to the sample, co-
immobilizing to the sensing surface different capturing
molecules each capable of binding to a respective analyte

and analyte analogue, contacting the sample with the
immobilized surface, sequentially reacting the bound
analyte analogues with different reagents specifically
reacting with the respective analyte analogues, and
5   determining the binding of each reagent to the respective
analyte analogue.

4.   The method according to claim 1, characterized in that
it comprises providing a number of samples corresponding to
10   the number of analytes to be determined, adding to each
sample a specific binding partner to a respective analyte
in the sample, co-immobilizing the analytes or analyte
analogues to the sensing surface, sequentially contacting
the respective samples with the immobilized surface, and
15   determining the binding of each specific binding partner to
the respective immobilized analyte or analyte analogue.

5.   The method according to claim 1, characterized in that
it comprises adding specific binding partners to the
20   respective analytes to the sample, co-immobilizing the
analytes or analyte analogues to the sensing surface,
contacting the sample with the immobilized surface,
sequentially reacting the bound specific binding partners
with respective specific reagents thereto, and determining
25   the binding of each specific reagent to the respective
specific binding partner.

6.   The method according to claim 1, characterized in that
it comprises co-immobilizing the analytes or analyte
30   analogues to the sensing surface, reacting the immobilized
analytes or analyte analogues with respective specific
binding partners such that substantially no unbound
analytes or analyte analogues remain on the surface,
contacting the sample with the surface to partially
35   displace bound specific binding partners therefrom,
sequentially reacting the remaining specific binding
partners with respective specific reagents, and determining

the binding of each reagent to the respective specific
binding partner.

7.   The method according to any one of claims 1 to 6,
characterized in that said mass change is determined as a
change in refractive index.

8.   The method according to claim 7, characterized in that
said change in refractive index is determined by an
evanescent wave sensing method, preferably surface plasmon
resonance.

9.   The method according to any one of claims 1 to 8,
characterized in that two different analytes are
determined.

10.   The method according to any one of claims 1 to 9,
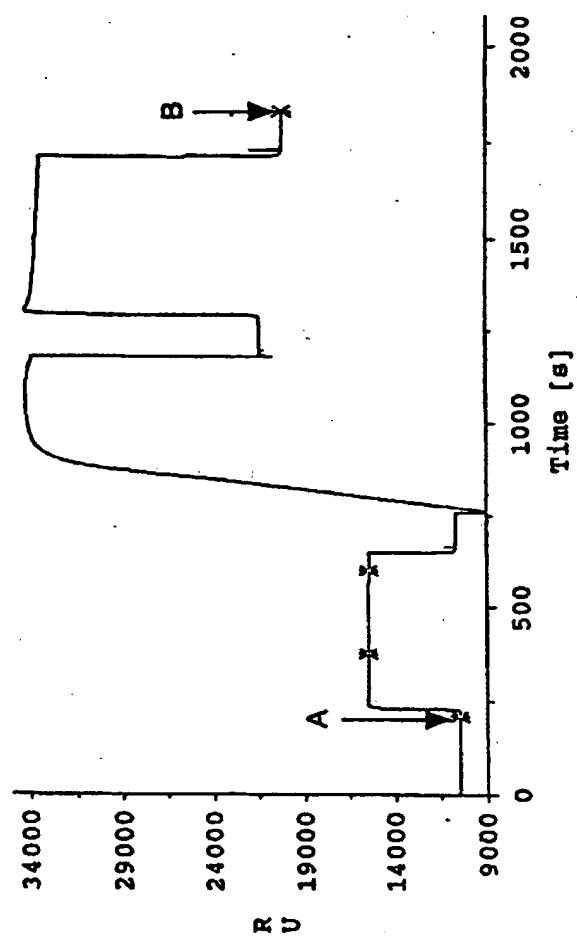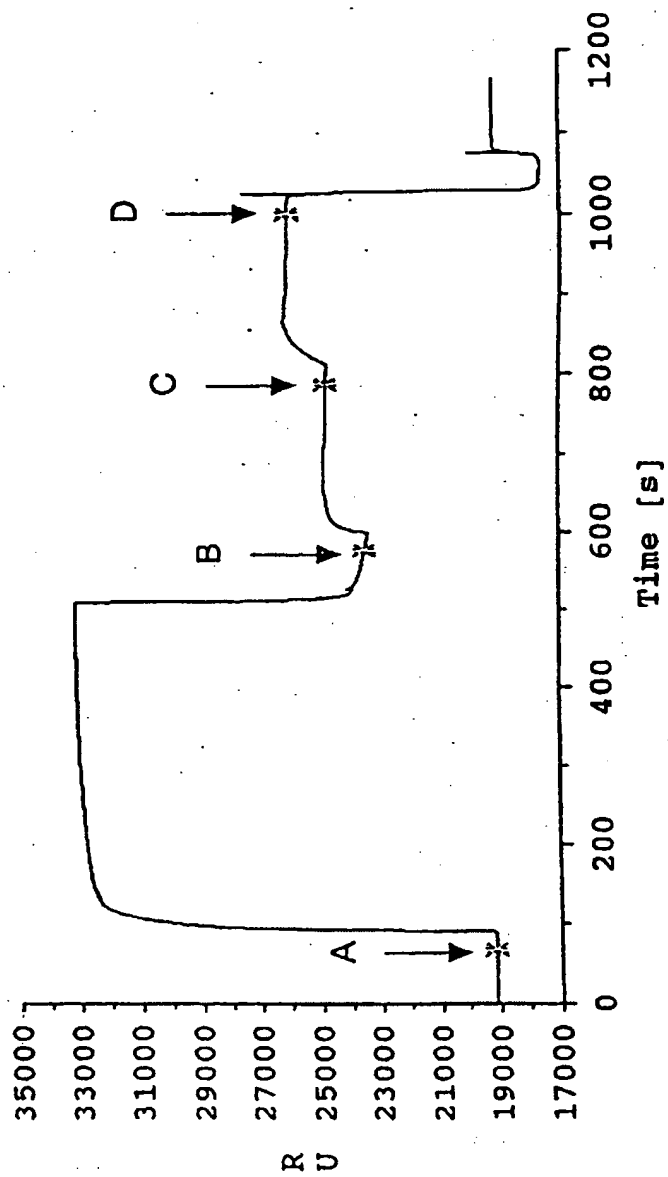characterized in that the assay is performed in a flow
cell.

1/7

FIG. 1

2/7

FIG. 2

FIG. 3

4 / 7

FIG. 4

FIG. 5
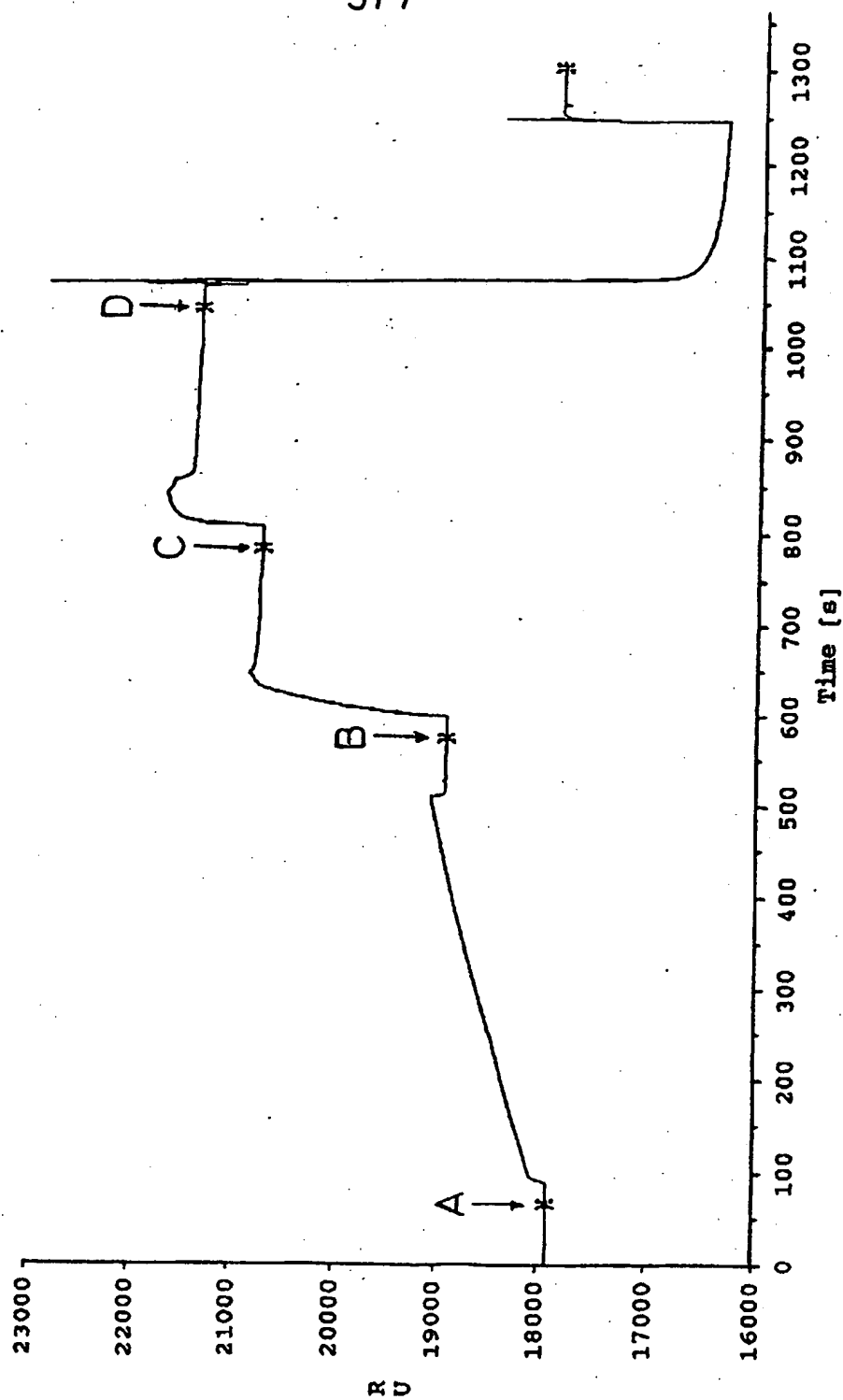
FIG. 6

7 / 7
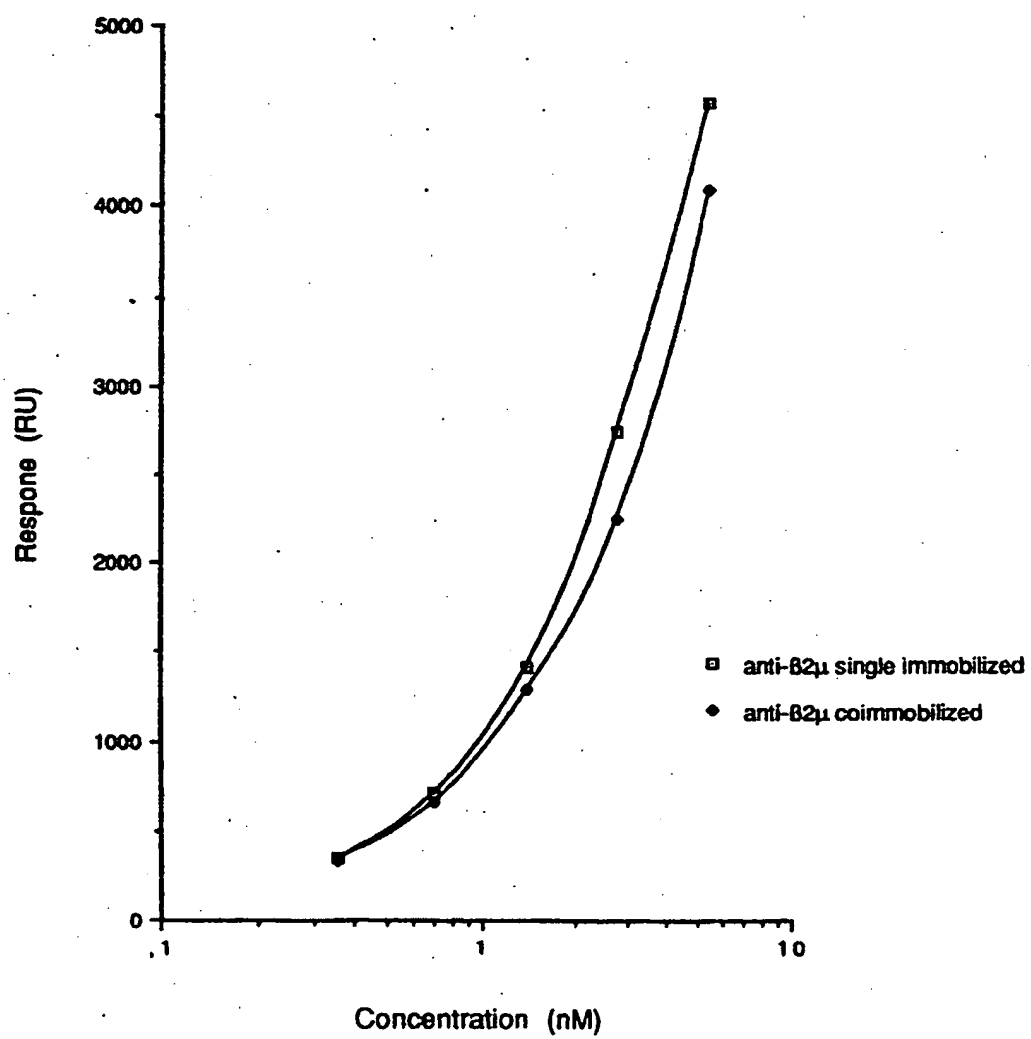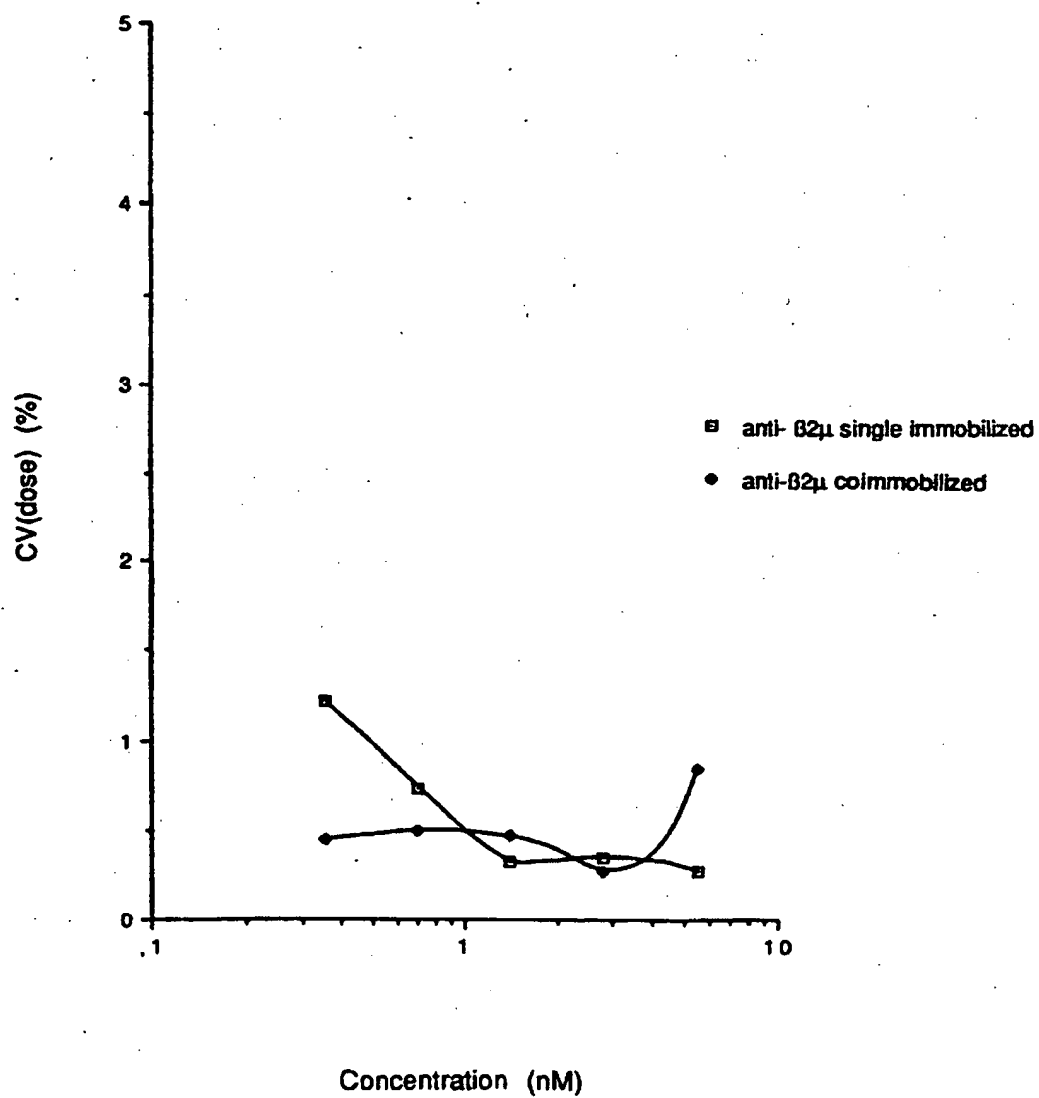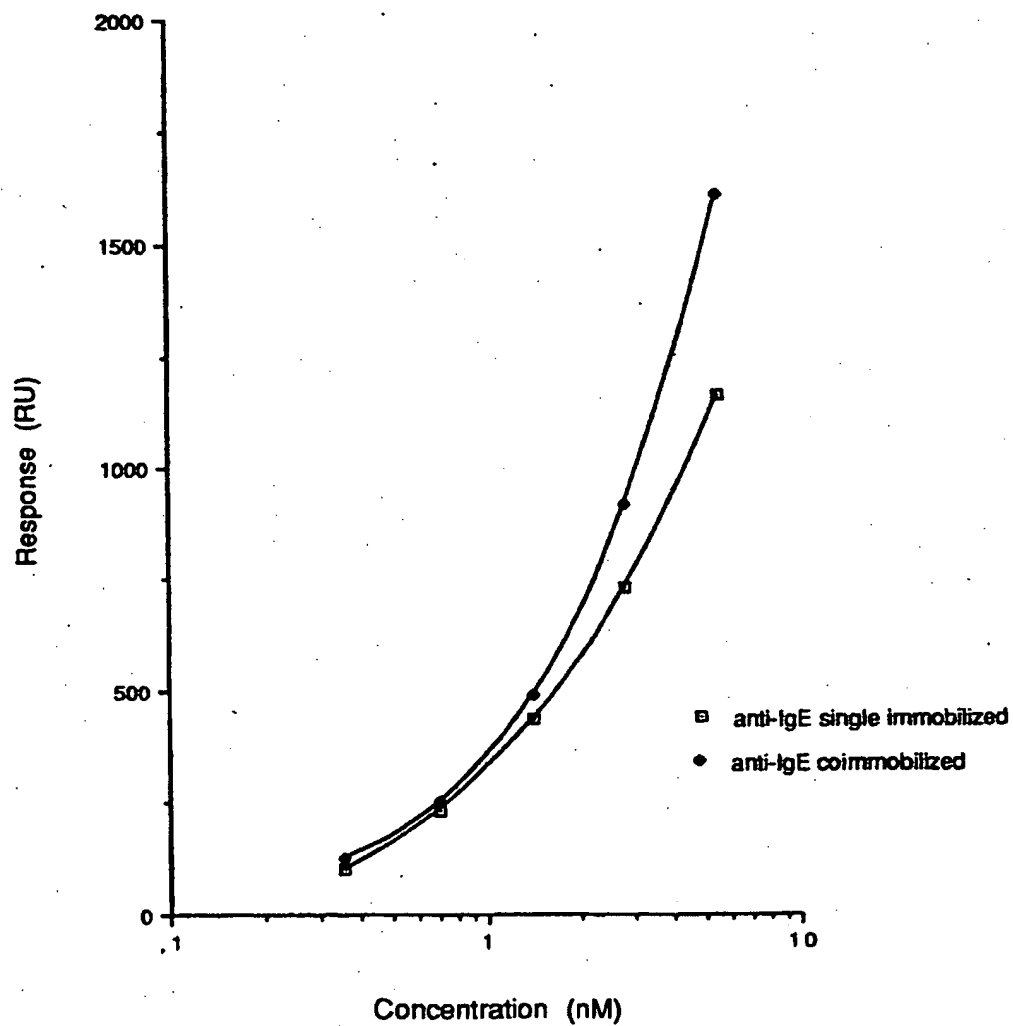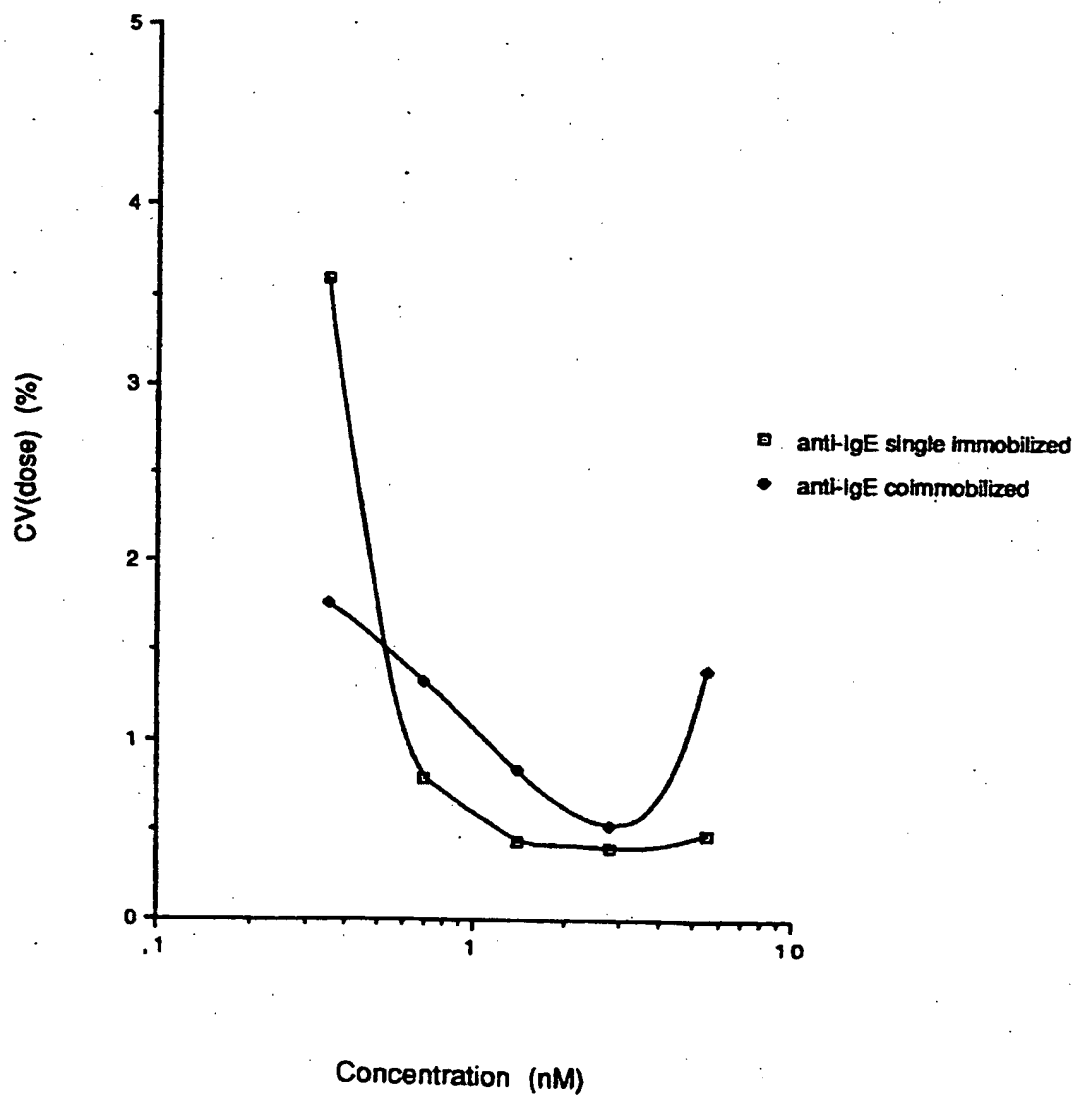
FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.	
PCT/SE 93/00488	

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: G01N 33/543, C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: G01N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CA, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A1, 9005306 (PHARMACIA AB), 17 May 1990 (17.05.90), page 17 - page 18"Subclass determination"	1-10
A	WO, A1, 9005303 (PHARMACIA AB), 17 May 1990 (17.05.90), page 18 III.1"Determination s of..."and Fig 3	1-10
A	WO, A1, 8002201 (MINNESOTA MINING AND MANUFACTURING COMPANY), 16 October 1980 (16.10.80), example 5	1-10

[X]	Further documents are listed in the continuation of Box C.	[X]	See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
17 Sept. 1993	20 -09- 1993

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 93/00488

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	DD, A1, 272134 (HUMBOLDT-UNIVERSITÄT ZU BERLIN), 27 Sept 1989 (27.09.89), page 2 - page 3, figure 3, claim 2	
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INTERNATIONAL SEARCH REPORT
Information on patent family members

26/08/93

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		EP-A-	0442930	28/08/91
WO-A1- 9005303	17/05/90	JP-T-	4501605	19/03/92
		SE-B,C-	462454	25/06/90
		SE-A-	8804073	10/11/88
WO-A1- 8002201	16/10/80	AU-B-	538366	09/08/84
		AU-A-	5993480	22/10/80
		CA-A-	1131341	07/09/82
		EP-A,B-	0026215	08/04/81
		SE-T3-	0026215	
US-A- 4315907	16/02/82	AU-B-	519877	24/12/81
		AU-A-	5218679	08/05/80
		CA-A-	1128856	03/08/82
		DE-A,C-	2943648	14/05/80
		FR-A,B-	2440556	30/05/80
		GB-A,B-	2034466	04/06/80
		JP-A-	55085252	27/06/80
		NL-A-	7907939	02/05/80
		SE-A-	7908936	01/05/80
EP-A2- 0402757	19/12/90	DE-A-	3919810	20/12/90
DD-A1- 272134	27/09/89	NONE		
DD-A5- 282003	29/08/90	NONE		